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Effects of polar carotenoids on the shape of the hydrophobic barrier of phospholipid bilayers

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Abstract

The value of A_z (z-component of the hyperfine interaction tensor) obtained directly from X-band EPR spectra of stearic acid spin labels and tempocholine dipalmitoylphosphatidic acid ester in frozen suspension of phosphatidylcholine (PC) membranes has been used as a hydrophobicity parameter. Using probes with the nitroxide moiety at various depths in the membrane, the shape of the hydrophobic barrier, which is determined by the extent of water penetration into the membrane, has been estimated. Incorporation of 10 mol% polar carotenoids, zeaxanthin, violaxanthin, or lutein into the saturated PC bilayer significantly increases the hydrophobicity of the membrane interior but decreases hydrophobicity (increases water penetration) in the polar headgroup region. Hydrophobicity at the membrane center increases from the level of propanol–pentanol, which have dielectric constants of 10–20, to the level of dipropylamine, with a dielectric constant close to 3. Longer alkyl chains decrease the effect of polar carotenoids in the polar headgroup region, but not in the central hydrophobic region. In an unsaturated egg yolk PC membrane, polar carotenoids were found to increase the hydrophobicity of the membrane interior to a higher level than in saturated PC membranes. At the membrane center hydrophobicity reaches the level close to pure hexane ($\epsilon \simeq 2$). The above results were confirmed by studying accessibility of $Fe(CN)_6^{3-}$ ion dissolved in water into dimyristoyl-PC-lutein membranes at 30°C. Obtained hydrophobicity profiles correlate well with permeability data for water in the literature. © 1998 Elsevier Science B.V.

Keywords: Zeaxanthin; Violaxanthin; Lutein; Membrane hydrophobicity; Water penetration; Spin label

1. Introduction

Carotenoids that do not show provitamin A activity still show biological and pharmaceutical activity [1], which is primarily attributed to their physical and chemical properties. These properties are determined by the carotenoid molecule's long, highly conjugated system of double bonds. For the effective physical interaction of the carotenoid molecule with lipids in a lipid bilayer membrane, the presence of polar groups on both ends of the long, stick-like molecule as well

Abbreviations: CW, continuous wave; DLPC, L-α-dilauroylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; EPR, electron paramagnetic resonance; PC, L-α-phosphatidylcholine; SASL, stearic acid spin label; *n*-SASL, *n*-doxylstearic acid spin label ($n = 5$, 9, 12, or 16); T-PC, tempocholine dipalmitoylphosphatidic acid ester

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as the ratio of the carotenoid molecule length to the membrane thickness is also very important. Rohmer et al. [2] suggested that these properties of carotenoid molecules were recognized during molecular evolution in using them as membrane reinforcers in bacteria. This prediction was confirmed by experimental work showing that polar carotenoids enhance the rigidity and reduce the water permeability of model lipid bilayers and reconstituted membranes from total lipids of *Halobacterium cutirubrum* [3–5].

Polar carotenoids are not only present in bacterial membranes but are selectively accumulated in some biological structures of eukaryotic organisms and used to perform specific biological processes. The polar carotenoids, lutein and zeaxanthin, are found in human macula [6–8]. They are selectively accumulated in the macula from the blood in the substantial range of 5–40 ng of each pigment [9]. Nonpolar carotenoids such as ß-carotene and lycopene are absent in the retina [9,10]. Polar carotenoids are also present transiently in the lipid bilayer portion of the thylakoid membranes where, during the xanthophyll cycle, they regulate membrane fluidity [11]. Recently, it has been recognized that polar carotenoids in photosynthetic apparatus stabilize the native structure of pigment-protein complexes [12–15].

We have previously used electron paramagnetic resonance (EPR) spin label techniques to investigate the effects of the polar carotenoids, zeaxanthin, violaxanthin, and lutein, on the organization of the lipid molecules in the membrane and their alkyl chain motion [16–19] as well as on oxygen transport within and across the lipid bilayer [16,20]. The major results that are related to the present work are summarized here: (1) polar carotenoids increase the order and decrease the alkyl chain motion in fluid-phase membranes, and disorder lipids in gel-phase membranes; (2) they increase mobility of the lipid polar headgroups; (3) the relationship of the length of the carotenoid molecule to the membrane thickness is a significant factor determining the effects of polar carotenoids on membrane fluidity; (4) the presence of unsaturated alkyl chains in membrane phospholipids moderates the effects of polar carotenoids; (5) polar carotenoids reduce the oxygen transport at all locations in the membrane.

In the present work, we extended our EPR study on the effect of polar carotenoids on the hydrophobicity profiles of model membranes. The hydrophobicity of the membrane interior determines not only the barrier of the membrane to permeation of polar molecules [4,21–27], but also the extent (depth) of ion penetration into the lipid bilayer [28–32]. A highly hydrophobic environment is also necessary to facilitate the energy transfer from light-harvesting systems to reaction centers in photosynthesis [14].

2. Materials and methods

2.1. Materials

All phosphatidylcholines (PCs) were purchased from Sigma (St. Louis, MO) and stearic acid spin labels (SASLs) from Molecular Probes (Eugene, OR). Tempocholine dipalmitoylphosphatidic acid ester (T-PC) was a generous gift from Dr. S. Ohnishi (Kyoto University, Japan). Lutein was purchased from Kemin Industries (Des Moines, IA) and the other carotenoids, zeaxanthin, and violaxanthin were extracted from fresh nettle leaves. The extract was saponified with KOH by the ''cold'' method [33] and separation was performed as previously described [18]. The visible absorption spectra of both carotenoids agreed with those known from the literature and did not show any features of *cis*-isomerization. The concentration of carotenoids has been determined spectrophotometrically using the extinction coefficients in ethanol: zeaxanthin, $\lambda_{max} = 450$ nm, $\epsilon_{max} = 144{,}300\ M^{-1}\ cm^{-1}$; violaxanthin, $\lambda_{max} = 440$ nm, $\epsilon_{max} = 153{,}000\ M^{-1}\ cm^{-1}$; lutein, $\lambda_{max} = 445$ nm, $\epsilon_{max} = 144{,}800\ M^{-1}\ cm^{-1}$.

2.2. Membrane preparation

The membranes used in this work were a multilamellar dispersion of investigated PC containing 1 mol% of spin label in the absence or presence of 10 mol% polar carotenoids, zeaxanthin, violaxanthin or lutein. Membranes were prepared by the following method of Subczynski et al. [17]. Chloroform solutions of the lipids, carotenoids, and spin labels were mixed (containing 0.5×10^{-5} mol of total lipids), and the chloroform was evaporated with a stream of nitrogen gas and then further evaporated under re-

duced pressure (0.1 mm Hg) for at least 12 h. A buffer solution (0.05 ml) was added to the dried lipids at about 20°C above the phase transition temperature of the PC membranes and vortexed vigorously. The buffer (0.1 M borate, pH 9.5) was used to ensure that all SASL probe carboxyl groups were ionized in the PC membranes [34]. The lipid dispersion was centrifuged briefly at $12{,}000 \times g$ for 15 min at 4°C and the loose pellet (about 20% lipid w/w) was used as a sample.

It is highly probable that in our preparations, part of the carotenoids forms small aggregates or micelles in the water phase of the suspension [4]. However, carotenoid aggregates and micelles remain outside of the lipid bilayer, and should not influence the EPR spectra that come from lipid-soluble spin labels. The amount of carotenoid that remains in the water phase depends on the method of liposome preparation and is rather high when solvents such as tetrahydrofuran, ethanol, or diethylether are mixed with an aqueous buffer solution [3,4]. We determined spectrophotometrically that in our preparations (buffer solution was added directly to the dry film of the PC-carotenoid mixture) about 97% of the carotenoid added remains incorporated into the lipid bilayer. Less than 3% forms micelles and microaggregates in the water phase. This evaluation was possible because after centrifugation, the aqueous phase is well separated from the liposomes, which form the pellet on the bottom of the tube in the case of DLPC, DMPC, DPPC, and DSPC membranes, or a liposome layer on the water surface in the case of EYPC membranes. In the Appendix, we present experimental evidence that in the presented work the incorporation ratios of polar carotenoids into all studied PC membranes are the same (or very close) and that the concentration of polar carotenoids applied (10 mol%) do not exceed the threshold values reported for the pigment miscibility within the fluid-phase PC membranes.

2.3. EPR measurements

All samples were run in capillaries made of the methylpentene polymer TPX (0.7 mm i.d.). The capillary was placed inside the EPR dewar insert and equilibrated with nitrogen gas, which was used for temperature control. The sample was thoroughly deoxygenated at a temperature above the phase transition of the lipid membrane. The sample was then frozen (−150°C) and EPR spectra were obtained at X-band with a Varian E-3 or E-109 spectrometer. The temperature was monitored using a copper-constantan thermocouple placed in the sample just above the active volume of the cavity. For continuous wave (CW) saturation measurements, the thermocouple was located in the sample at the center of the microwave cavity. CW saturation measurements were carried out at 30°C. Special care was taken to control the sample temperature, its position in the spectrometer cavity, and water content [35]. All preparations and measurements of samples with carotenoids were performed in the dark or in dim light. The chemical structures of polar carotenoids and spin labels, and their approximate location in the lipid bilayer have been previously reported [17,19].

2.4. $Fe(CN)_6^{3-}$ accessibility parameter

An experimental power saturation curve, the plot of the first derivative peak-to-peak amplitude of the EPR signal (Y') versus the square root of an incident power ($\sqrt{P}$), can be fitted to the theoretical description of power saturation behavior for the homogenous Lorentzian line [36]:

$$Y' = A\frac{\sqrt{P}}{(1+cP)^{3/2}}. \qquad (1)$$

Here A is a normalizing factor and c is a parameter proportional to the T_1T_2 product. T_1 and T_2 are the spin–lattice and spin–spin relaxation times. The experimental data were fitted to Eq. (1) using the best-fit procedure with c and A as the adjustable parameters.

During bimolecular collisions of the nitroxide fragment with transition metal ions the dominant magnetic interaction is Heisenberg exchange [37]. As a result, T_1 of the nitroxide is shortened. By analogy with the oxygen transport parameter [38] and with the copper transport parameter [39,40], the $Fe(CN)_6^{3-}$ accessibility parameter is defined as:

$$W\left(Fe(CN)_6^{3-};x\right) = T_1^{-1}\left(50\,\text{mM}\,K_3Fe(CN)_6;x\right) - T_1^{-1}\left(\text{no}\,K_3Fe(CN)_6;x\right) \qquad (2)$$

Here $W(Fe(CN)_6^{3-};x)$ is a function of both the concentration and the translational diffusion coefficient of $Fe(CN)_6^{3-}$ at the ''depth'' x in the lipid bilayer when the $K_3Fe(CN)_6$ concentration in the buffer is 50 mM.

T_1T_2 product can be obtained from CW saturation experiments using conventional EPR spectrometers. In principle, the collisions with the paramagnetic ions should also decrease spin–spin relaxation time T_2 of the nitroxide. However, for the system investigated here $T_2 < T_1$ and the $Fe(CN)_6^{3-}$ concentration within the lipid bilayer is very small. Thus, T_2 remains essentially unchanged by the presence of $K_3Fe(CN)_6$ in the buffer and the effect on T_1 will dominate. Eq. (2) should be then transformed to a new form, which allows calculation of the $Fe(CN)_6^{3-}$ accessibility parameter directly from the T_1T_2 product obtained from the CW saturation measurements and fitting procedure:

$$W\left(Fe(CN)_6^{3-};x\right) = \frac{[T_1T_2(50\,mM\,K_3Fe(CN)_6;x)]^{-1} - [T_1T_2(no\,K_3Fe(CN)_6);x]^{-1}}{\Delta H_{pp}(x)} \quad (3)$$

T_1T_2 (50 mM $K_3Fe(CN)_6$; x) and T_1T_2 (no $K_3Fe(CN)_6$; x) are T_1T_2 products, respectively, in the presence and absence of 50 mM $K_3Fe(CN)_6$ in the buffer for the particular spin label in the membrane. This equation allows comparison of the $Fe(CN)_6^{3-}$ accessibility parameters between different spin labels with different spectral linewidth, because the accessibility parameter is normalized with respect to the linewidth:

$$\Delta H_{pp}(x)\,\alpha\,T_2^{-1}\left(no\,K_3Fe(CN)_6;x\right) = T_2^{-1}\left(50\,mM\,K_3\,Fe(CN)_6;x\right) \quad (4)$$

which corresponds to the peak-to-peak linewidth of the central line in the EPR spectrum $\Delta H_{pp}(x)$, for non-saturating conditions in the absence of $K_3Fe(CN)_6$. Similar data processing was proposed by Snel and Marsh [29]. In the presented results, the $Fe(CN)_6^{3-}$ accessibility parameter was obtained as defined in Eq. (3) with c in the place of the T_1T_2 product.

3. Results and discussion

3.1. Hydrophobicity profiles of saturated PC membranes

The local hydrophobicity in the membrane was monitored primarily using A_z (z-component of the hyperfine interaction tensor of the nitroxide spin label) as a conventional experimental observable [30,41]. A_z can be immediately obtained from EPR spectra of spin labels measured for a frozen suspension of membranes. With an increase in solvent polarity, A_z increases. In this type of work, a nitroxide free-radical is placed at various depths in the lipid bilayer and hydrophobicity profiles across membranes are obtained. Griffith et al. [41] demonstrated that hydrophobicity in the membrane is largely determined by the extent of water penetration into the membrane, since dehydration abolishes the hydrophobicity gradient in liposome samples. In our present and previous [30] studies we related hydrophobicity as observed by A_z at a selected depth in the membrane to hydrophobicity (or ϵ) of bulk organic solvents.

In the present work, dilauroyl-PC (DLPC), dimyristoyl-PC (DMPC), dipalmitoyl-PC (DPPC) and distearoyl-PC (DSPC), containing 12, 14, 16, and 18 carbons per alkyl chain, respectively, were used to investigate the effect of alkyl chain length. Fig. 1 shows the hydrophobicity profiles across saturated PC membranes in the absence and presence of 10 mol% polar carotenoids. Effects of alkyl chain length on hydrophobicity profiles in pure lipid bilayers have been described previously [30]. They can be summarized as follows. Saturated PC membranes show low hydrophobicity even at the center, comparable to those of propanol and octanol, which have dielectric constants of 10–20 (see Fig. 2 and its explanation in [30] to relate the $2A_z$ value with the dielectric constant of a bulk solvent; readers are warned not to overinterpret this comparison). Longer alkyl chains make the central hydrophobic region wider without increasing the level of hydrophobicity.

Incorporation of 10 mol% polar carotenoids (zeaxanthin, violaxanthin or lutein) causes a considerable increase in hydrophobicity in the central region of the bilayer, which is deeper than the fifth carbon in the alkyl chain. At the center, hydrophobicity reaches the level of dipropylamine ($\epsilon \simeq 3$). The presence of polar carotenoids induces an increase in polarity in the headgroup region. The effect is strong for DLPC and DMPC bilayers, weak for the DPPC bilayer, and not observable for the DSPC bilayer. The hydrophobicity profiles across DLPC, DMPC, and DPPC bilayers obtained in the presence and absence of polar

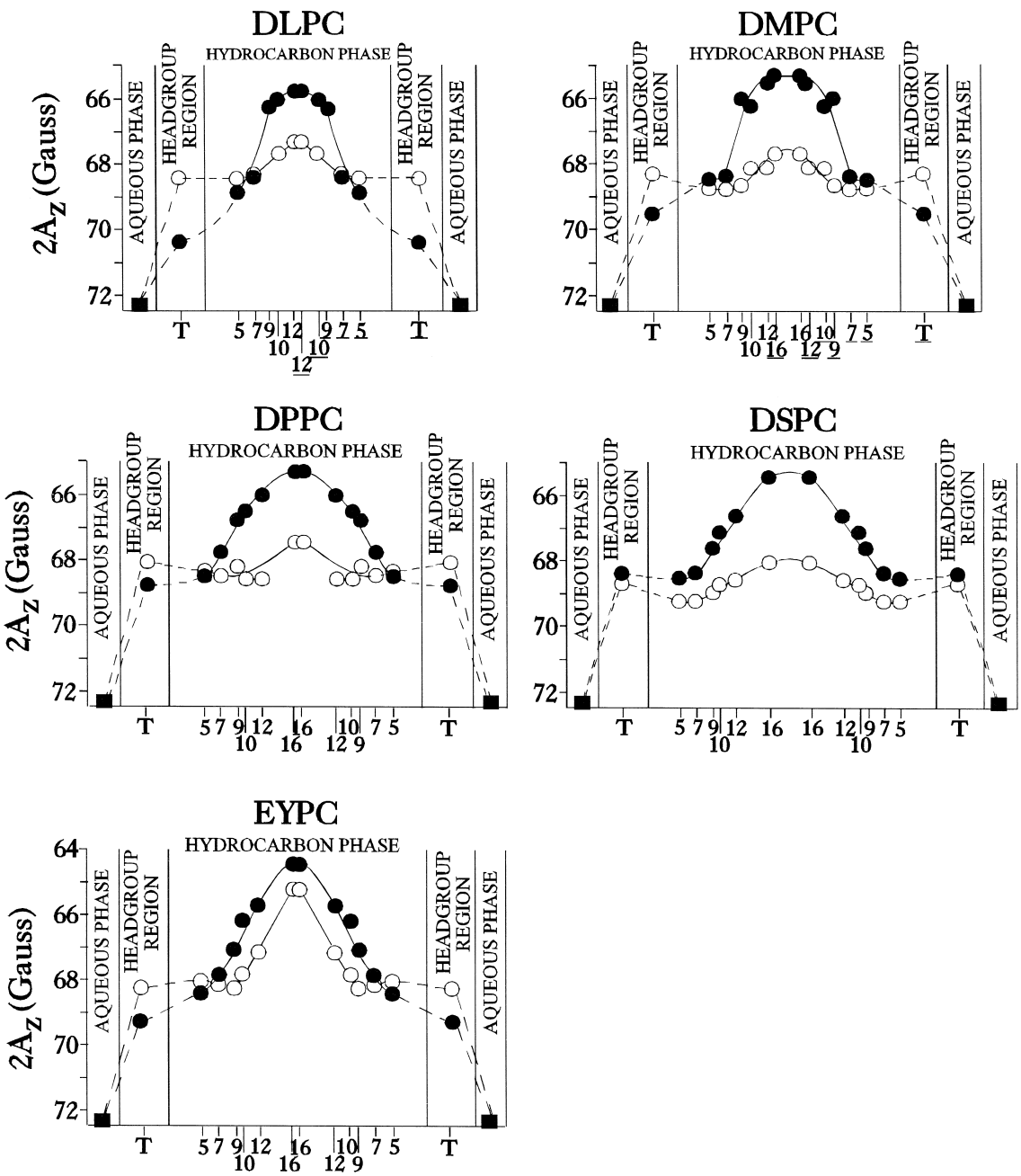

Fig. 1. Hydrophobicity profiles ($2A_z$) across PC membranes in the absence (○) and presence (●) of 10 mol% zeaxanthin. Upward changes indicate increases in hydrophobicity. The data for DLPC, DMPC, DPPC, DSPC (saturated PC) and EYPC (unsaturated PC) membranes are shown. Approximate locations of the nitroxide moieties of spin labels are indicated by numbers under the baseline. The underlined numbers for *n*-SASL in DLPC and DMPC indicate that these SASLs are intercalated in the right half of the bilayer (SASLs are longer than the host phospholipid alkyl chains). T indicates T-PC. $2A_z$ for SASL in the aqueous phase was calculated from the isotropic hyperfine constant as described previously [30]. Broken lines are used for connecting the points obtained with different types of the nitroxide radicals. The major features of the profiles were also obtained in the presence of 10 mol% violaxanthin or lutein and are identical to those obtained for zeaxanthin.

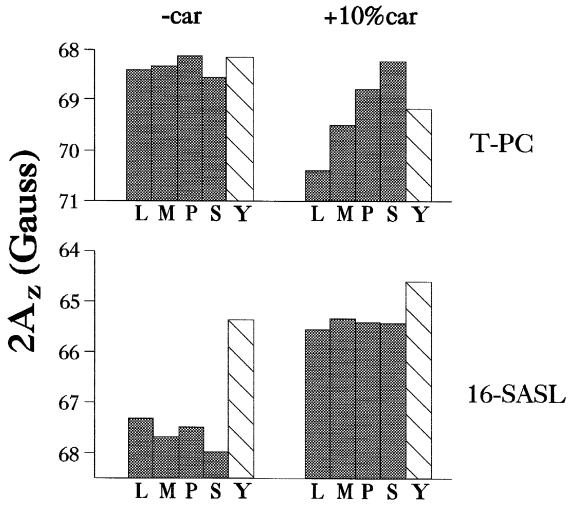

Fig. 2. $2A_z$ values (in gauss) are shown for membranes without polar carotenoids (left column) and with 10 mol% zeaxanthin (right column) measured with T-PC and 16-SASL. Taller bars indicate higher hydrophobicity. Note that the absolute scales for $2A_z$ are different for each row. T-PC is a probe for the near-surface region of the membrane, while 16-SASL is a probe for the center of the membrane. Symbols: L, DLPC; M, DMPC, P, DPPC; S, DSPC; Y, EYPC; dark bars, PCs with saturated alkyl chains; hatched bars, PC with *cis* unsaturation. For DLPC, hydrophobicity in the membrane center is measured with 12-SASL.

carotenoids cross each other in the near headgroup region at about the C5 position. Effects in and near the headgroup regions depend on membrane thickness. In the DSPC bilayer, however, hydrophobicity in the presence of polar carotenoids is higher at all locations in the membrane.

3.2. Hydrophobicity profiles of unsaturated PC membranes

As a model of biological membranes with mixed and unsaturated alkyl chains, bilayer membranes made of egg yolk PC (EYPC) were studied. Approximately, 70% of EYPC is 1-palmitoyl-2-oleoyl-PC [42]. The hydrophobicity profiles of those membranes in the absence and presence of 10 mol% polar carotenoids are shown in Fig. 1 (bottom part). We should compare the hydrophobicity profiles of EYPC with those of DPPC because these two membranes have similar thicknesses of the glycerohydrocarbon interior (29 and 29.3 Å, respectively) [43]. As reported earlier [30], introduction of unsaturation into a hydrocarbon chain increases hydrophobicity at all locations in the hydrocarbon region of the membrane. The hydrophobicity in the membrane center reaches approximately that of dipropylamine with a dielectric constant close to 3. Incorporation of 10 mol% polar carotenoids causes a small increase in polarity of the headgroup and near headgroup (C5 position) regions, and an increase in hydrophobicity at deeper locations in the membrane. In the bilayer center hydrophobicity increases up to the level close to pure hexane ($\epsilon \simeq 2$). Since the level of hydrophobicity in the middle of the bilayer of pure EYPC membrane is already high, the polar carotenoid-induced increment in the central region is much smaller than in saturated membranes. In both saturated and unsaturated PC membranes containing polar carotenoids, the hydrophobicity profiles have a characteristic bell-shape. For thick PC membranes, polar carotenoids decrease the extent of water penetration into the entire hydrocarbon region.

3.3. Different effects of polar carotenoids in headgroup regions and central regions of PC membranes

Fig. 2 summarizes the effect of 10 mol% polar carotenoids on a variety of PC membranes at the membrane surface and in the middle of the bilayer, as probed by T-PC and by 16-SASL, respectively. Observations with T-PC indicate that polar carotenoids decrease hydrophobicity near the membrane surface. The effect decreases as the membrane thickness increases. The carotenoid-induced decrease is the largest for DLPC (containing 12 carbon alkyl chains) and disappears for DSPC (containing 18 carbon alkyl chains) indicating strong dependence on the membrane thickness. The effect in EYPC is similar to that in DPPC, showing that membrane thickness, not the degree of the alkyl chain unsaturation, is the major factor in determining the disturbance caused by polar carotenoids in the polar headgroup region.

Intercalation of polar carotenoids into the PC bilayer with polar groups located between PC headgroups causes a separation of the headgroups and increases water penetration into that region. Similarly, cholesterol [30] and gramicidin [32] increase water accessibility into the membrane headgroup region. However, the effect of cholesterol is always the

same, independent of the membrane thickness. This difference results from the different location of cholesterol and polar carotenoid in the membrane. Cholesterol is located in one half of the bilayer, floating under the membrane surface. The polar hydroxyl group anchors the cholesterol molecule at the membrane surface [44] with small vertical displacement (sinking into the membrane) for DLPC and dioleoyl-PC, and at high cholesterol contents [30]. One polar carotenoid molecule crosses the lipid bilayer, and its two polar groups interact with opposite hydrophilic surfaces of the membrane [45]. An increase in membrane thickness causes the polar groups of the carotenoid molecule to sink deeper into the hydrocarbon region. Contact between PC's headgroups is restored (they ''close'' above the carotenoid molecule), decreasing water penetration into that region. As we have previously shown [18], the effects of polar carotenoids on saturated PC bilayer order and motion also depend on the membrane thickness.

16-SASL indicates that in the presence of 10 mol% polar carotenoids, the level of hydrophobicity is very similar in all saturated PC membranes independent of membrane thickness, and the induced hydrophobicity increase is significant (dielectric constant decrease from the value of 10–20 to a value close to 3). In the unsaturated EYPC bilayer, the induced hydrophobicity increase is small but the final level of hydrophobicity in the presence of 10 mol% polar carotenoids reaches the value close to that of pure hexane ($\epsilon \simeq 2$). We think that the effect of polar carotenoids in the hydrocarbon region of the bilayer is a result of the introduction of large amounts of conjugated double bonds into that region. It has been shown that lipid bilayers containing unsaturated alkyl chains demonstrate less water penetration into the hydrocarbon region than saturated bilayers [30,46]. It is also known that unsaturated hydrocarbons are less hygroscopic than saturated hydrocarbons.

3.4. Ion penetration into the membrane

Our measurements show that polar carotenoids increase hydrophobicity (decrease water penetration) of the central region in the membrane. The effect is especially pronounced in saturated lipid bilayers independent of the membrane thickness. Higher hydrophobicity should also decrease penetration of other small polar molecules into the same region. For this reason, we investigated the magnetic interaction of $Fe(CN)_6^{3-}$ ion dissolved in water with SASLs in the DMPC membrane using the CW power-saturation technique. Fig. 3 shows representative experimental power-saturation data for 12-SASL in the DMPC bilayer in the presence and absence of both lutein and $K_3Fe(CN)_6$ at 30°C, together with fitted curves to Eq. (1). It is evident that the effect of $Fe(CN)_6^{3-}$ on power-saturation of 12-SASL is greater in the absence of lutein. Without $K_3Fe(CN)_6$, the power-saturation curves are very similar in the presence and absence of lutein. The addition of polar carotenoids (lutein) to fluid-phase model membranes increases T_1 and decreases T_2 (increases linewidth) of SASL spin labels [17,19], which is a probable explanation for the negligible effect of lutein on CW saturation curves in the absence of $K_3Fe(CN)_6$.

From CW saturation curves the $Fe(CN)_6^{3-}$ accessibility parameters for 5-, 9-, and 12-SASL in DMPC (14 carbon alkyl chains) membranes at 30°C were obtained in the presence and absence of 10 mol%

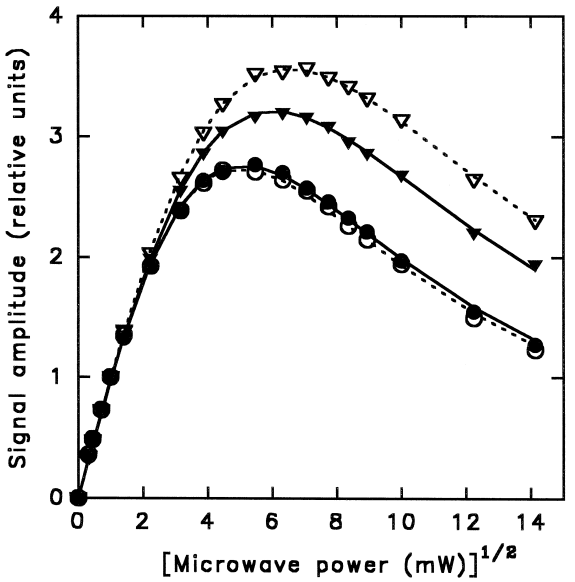

Fig. 3. CW saturation data for the central line of 12-SASL in DMPC membranes at 30°C. Membranes without (○, ∇) and in the presence (●, ▼) of 10 mol% lutein, in the absence (○, ●) and presence (∇, ▼) of 50 mM $K_3Fe(CN)_6$ in the buffer. In the absence of $K_3Fe(CN)_6$, 300 mM NaCl was added instead. The dashed and solid lines show the results of the best-fit according to Eq. (1) for membranes without and in the presence of 10 mol% lutein, respectively.

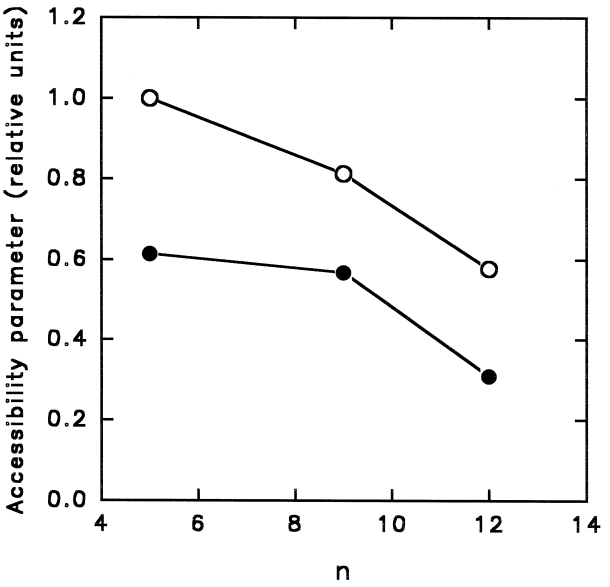

Fig. 4. Relative accessibility parameter obtained at 30°C in DMPC membranes and plotted as a function of the position of the nitroxide group of SASL in the membrane. Symbols: (○)- no polar carotenoids, (●)- 10 mol% lutein. All data were divided by the $Fe(CN)_6^{3-}$ accessibility parameter for 5-SASL in pure DMPC membranes.

lutein, and are shown in Fig. 4. The higher value of the accessibility parameter indicates better penetration of $Fe(CN)_6^{3-}$ ions into the membrane depth at which the nitroxide moiety is located. In the absence and presence of lutein, penetration of $Fe(CN)_6^{3-}$ gradually decreases towards the membrane center. In the presence of 10 mol% lutein, the level of penetration of $Fe(CN)_6^{3-}$ decreases by about 45% in the membrane center and by about 35% in the near polar headgroup region. These results obtained in fluid-phase membranes are consistent with the hydrophobicity profiles presented in Fig. 1, showing that hydrophobicity profiles obtained at −150°C provide a good estimate of those at physiological temperatures (see also [30] for further evidence for this statement).

4. General discussion

The results obtained here are consistent with those obtained for permeability of water molecules across the membrane. Carotenoids with polar groups on both ends of the molecule (zeaxanthin, astaxanthin, and bacterioruberin) significantly decrease water permeability across the lipid bilayer of unilamellar vesicles made of saturated PCs (DMPC or diphytanyl PC) or made of total polar lipids of *Halobacterium* (diphytanylglyceryl ethers: a mixture of different polar headgroups) [3,5]. However, there was no measurable effect of zeaxanthin on water penetration across the unsaturated EYPC membrane [4]. Our data also show that the polar carotenoid-induced relative increase in the membrane hydrophobicity of the EYPC bilayer is rather small. Additionally, this slight increase is somewhat compensated by the increase in water penetration into the headgroup and near headgroup regions.

We think that the observed effects of polar carotenoids on membrane hydrophobicity may be of biological relevance:

1. It has been shown that membranes of extreme halophyles [47,48] and thermophilic bacteria [49,50] contain a fairly large amount of polar carotenoids. In red membranes of *Halobacterium cutirubrum* the concentration of carotenoids was estimated at about 5 mol% [5]. These bacteria, which live in extreme conditions, should possess stable membranes that provide a high barrier for nonspecific inward and outward permeation of small molecules. Incorporation of polar carotenoids into the membrane serves these purposes well. Carotenoids stabilize both halves of the lipid bilayer like transmembrane ''rivets,'' increase membrane rigidity by ordering the alkyl chains of lipids, and raise the membrane hydrophobic barrier for polar molecules and ions. An increase in hydrophobicity should greatly increase the activation energy required for polar small molecules to cross the membrane. Our results provide additional support to Rohmer's hypothesis that polar carotenoids regulate the membrane property in Procaryota in a manner similar to cholesterol in Eucaryota [2,30].
2. The initiation of lipid peroxidation, which requires hydrogen abstraction from unsaturated lipid carbon–carbon bonds and secondary initiation from lipid peroxides, is promoted by reactions with certain metal ions and water-soluble free radicals [51]. These reactions can occur at the membrane–water interface during vertical fluctuations of lipid alkyl chains toward the membrane surface [19,52].

They can also occur in the membrane core because metal ions and other polar molecules can, to a certain degree, penetrate the lipid bilayer [28–32]. A polar carotenoid-induced increase in membrane hydrophobicity decreases the penetration of metal ions and polar free radicals into the membrane, and thus, should be considered as an additional mechanism by which carotenoids prevent lipid peroxidation.

3. We also think that the property of polar carotenoids to increase local membrane hydrophobicity is significant to facilitate efficient energy transfer in photosynthesis. Carotenoids are present in light-harvesting complexes where they act as accessory light-harvesting pigment, prevent photodynamic destruction, and stabilize the native structure of pigment–protein complexes [15,53,54]. Recently, the crystal structure of the light-harvesting complex from photosynthetic bacteria has revealed that carotenoid molecules span the complex, forming a kind of ''barrel'' around the bacteriochlorophyll molecules [14]. In this way, carotenoids should provide a highly hydrophobic environment that reduces the dielectric constant and facilitates the delocalization of the excited state of bacteriochlorophyll molecules over the ring of bacteriochlorophylls. The energy is then available for an efficient transfer to the reaction center from any part of the ring, where it is ''trapped.''

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Appendix A. Incorporation of polar carotenoids into PC membranes

Most authors agree that polar dihydroxycarotenoids (zeaxanthin, violaxanthin, and lutein) are well dissolved in fluid-phase PC membranes, and an incorporation ratio of 10 mol% is possible. Values ranging from 9.5 mol% for EYPC membranes [4] and 10 mol% for DMPC membranes [3,4] to 17 mol% for DMPC membranes [55] have been reported. However, in Ref. [4], it was reported that in DPPC membranes the zeaxanthin incorporation ratio is not greater than 0.5 mol% because of the mismatch between the length of the carotenoid molecule (30.2 Å, the distance between oxygens in the dihydroxycarotenoid molecule) and the thickness of the hydrophobic region of the DPPC bilayer (40 Å, according to the evaluation presented in Ref. [4]). A careful reading of the ''Experimental Procedure'' section in this paper reveals that the authors made a mistake during preparation of DPPC liposomes. They prepared them at 25°C, a temperature well below the main phase transition temperature (41.6°C) and even below the pretransition temperature of DPPC membranes, which should dramatically affect zeaxanthin incorporation. Additionally, as evaluated in Ref. [4], the thicknesses of the hydrophobic region of PC bilayers differ significantly from those presented in the literature. This ''coincidence'' gives the impression that the excellent match of the length of zeaxanthin (30.2 Å) with the thickness of the hydrophobic region of the DMPC bilayer (30 Å) should facilitate zeaxanthin solubility, while a striking mismatch in the case of the DPPC bilayer (40 Å) should severely decrease its solubility. The evaluation of the membrane thicknesses in Ref. [4] is based on molecular mechanics models, taking into account an average 2 gtg kinks per chain in the fluid phase and measuring the length of hydrocarbon chains of DMPC and DPPC molecules. However, the hydrophobic length of dihydroxycarotenoids should be compared with the thickness of the hydrophobic region of the lipid bilayer. The thickness of the glycerohydrocarbon region (H) of a fully hydrated saturated PC membrane with 12–18 carbons in alkyl chains at a temperature well above its phase transition temperature can be calculated from the approximation derived by Cornell and Separovic [43]:

$$H = (18.7 + 0.66n)\,A. \quad (5)$$

Here n is the number of alkyl-chain carbons. They used X-ray diffraction data from other papers. The thicknesses obtained for DLPC, DMPC, DPPC, and DSPC membranes are 26.6, 27.9, 29.3, and 30.6 Å,

respectively. As it can be seen, the thicknesses of the glycerohydrocarbon regions are nearly the same for all PC membranes that were studied. On this basis, one would not expect the incorporation ratios of polar carotenoids into these bilayers to vary significantly. The major change in dimension that occurs with the variation in alkyl chain length is the area occupied per molecule rather than the bilayer thickness [43]. Also, the results of X-ray diffraction experiments and linear dichroism measurements [45] demonstrate that in fluid-phase DMPC membranes, zeaxanthin and violaxanthin molecules are inclined with respect to the normal to the bilayer surface because the thickness of the hydrocarbon membrane interior is less than the length of dihydroxycarotenoids. The best match of the length of the dihydroxycarotenoid molecule (30.2 Å) with the thickness of the hydrocarbon interior of the PC bilayer is for DPPC and DSPC membranes. For these membranes, the incorporation ratios of dihydroxycarotenoids should be the highest. This is confirmed by the data obtained by Kolev and Kafalieva [56] on the basis of calorimetric and optical measurements showing that zeaxanthin can be incorporated into fluid-phase DPPC membranes up to 28 mol%.

As we demonstrated earlier [17,18] and confirmed in the presented work, 10 mol% polar carotenoids (zeaxanthin, violaxanthin and lutein) affects the properties (fluidity, alkyl chain order, hydrophobicity) of saturated PC membranes with different alkyl chain length (from 12 to 22 carbons) differently in and near the headgroup region. Their effects on the membrane properties in the membrane center were, however, very similar independent of membrane thickness. These observations serve as evidence for similar solubility of dihydroxycarotenoids in saturated PC membranes with different alkyl chain length.

Additional data on the effect of different amounts of dihydroxycarotenoids (0 to 10 mol%, added to the sample during preparation) on DLPC, DMPC, DPPC, and DSPC membrane fluidity in the membrane center obtained by measurement of the rotational correlation time of 16-SASL are shown in Fig. 5. Comparison is made at 60°C, where all membranes are in the fluid phase. Fig. 6 shows the effect of different amounts of dihydroxycarotenoids (0–10 mol%, added to the sample during preparation) on the hydrophobicity of DLPC, DMPC, DPPC, and DSPC membranes in the

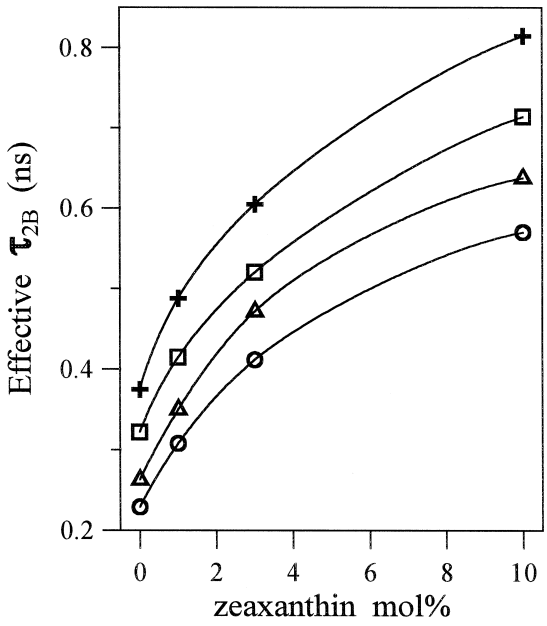

Fig. 5. Effective rotational correlation time of 16-SASL in DLPC (○), DMPC (△), DPPC (□), and DSPC (+) membranes plotted as a function of mole fraction of zeaxanthin at 60°C. Similar results were obtained for violaxanthin and lutein.

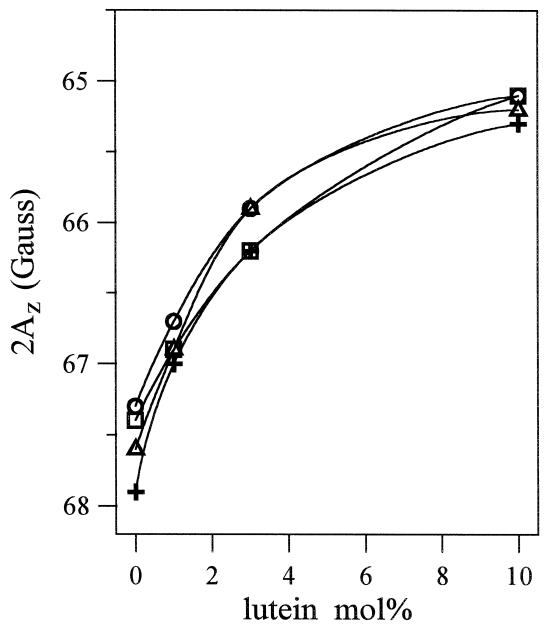

Fig. 6. $2A_z$ values for 16-SASL in DLPC (○), DMPC (△), DPPC (□), and DSPC (+) membranes plotted as a function of mole fraction of lutein. Upward changes indicate increases in hydrophobicity.

membrane center measured as $2A_z$ of 16-SASL. Both sets of curves demonstrate that polar carotenoids affect these membrane properties similarly in all membranes. The induced changes are proportional to the amount of carotenoids added with small signs of saturation only at 10 mol%. No discontinuity in measured properties was observed for lower carotenoid concentrations. On the basis of these experimental results and data from the literature indicating that polar carotenoids are miscible in the range of 0 to 10 mol% with DMPC membranes [3,4,55], EYPC membranes [4], and DPPC membranes [56], we concluded that in all saturated PC membranes that were investigated as well as in EYPC membranes, the observed effects are induced by the same (or very similar) amount of polar carotenoids incorporated into the lipid bilayers.

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